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Free Radicals Enhance Basal Release of D-[^{3}H]Aspartate from Cerebral Cortical Synaptosomes

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Abstract: Excessive generation of free radicals has been implicated in several pathological conditions. We demonstrated previously that peroxide-generated free radicals decrease calcium-dependent high K^+-evoked L-[^{3}H]-glutamate release from synaptosomes while increasing calcium-independent basal release. The present study evaluates the nonvesicular release of excitatory amino acid neurotransmitters, using D-[^{3}H]aspartate as an exogenous label of the cytoplasmic pool of L-glutamate and L-aspartate. Isolated presynaptic nerve terminals from the guinea pig cerebral cortex were used to examine the actions and interactions of peroxide, iron, and desferrioxamine. Pretreatment with peroxide, iron alone, or peroxide with iron significantly increased the calcium-independent basal release of D-[^{3}H]aspartate. Pretreatment with desferrioxamine had little effect on its own but significantly limited the enhancement by peroxide. High K^+-evoked release in the presence of Ca^{2+} was enhanced by peroxide but not by iron. These data suggest that peroxide increases nonvesicular basal release of excitatory amino acids through Fenton-generated hydroxyl radicals. This release could cause accumulation of extracellular excitatory amino acids and contribute to the excitotoxicity associated with some pathologies. **Key Words:** Hydroxyl free radicals—Amino acids—Neurotransmitter—Peroxide—Synaptosome—Iron—Glutamate—Aspartate.
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Biological systems produce free radicals as intermediate by-products of oxidative metabolism. Normally, these potentially hazardous compounds are maintained at a low level by the action of free radical scavengers and specific enzyme systems. However, under certain conditions, such as aging, ischemia, hyperoxia, or exposure to ionizing radiation, free radical levels can become abnormally high. Free radicals have been shown to have damaging effects on nervous system function (Halliwell and Gutteridge, 1984; Colton and Gilbert, 1985; Pellmar, 1986, 1987; Tolliver and Pellmar, 1987) and are thought to be involved in a number of neurological conditions (Halliwell and Gutteridge, 1984; Halliwell, 1992).

Free radicals generated by either γ-radiation or exposure to hydrogen peroxide decrease synaptic efficacy in hippocampal slices (Pellmar, 1986, 1987; Tolliver and Pellmar, 1987) and at the crustacean neuromuscular junction (Colton and Gilbert, 1985). Superoxide, generated from xanthine/xanthine oxidase also depresses glutamate-mediated synaptic potentials at lobster neuromuscular junction and squid giant synapse (Colton et al., 1991). Synaptosomal studies (Gilman et al., 1992) have demonstrated that peroxide decreases the depolarization-evoked calcium-dependent release of L-[^{3}H]glutamate neurotransmitter that might underlie the decrease in synaptic potentials. In addition, the synaptosomal studies revealed an increase in basal release of neurotransmitter with exposure to peroxide-generated free radicals (Gilman et al., 1992). A similar increase in basal release is observed with exposure of hippocampal slices to xanthine/xanthine oxidase (Pellegrini-Giampietro et al., 1990).

Hydroxyl free radicals can be generated through the Fenton reaction by interaction of hydrogen peroxide with tissue iron (Pellmar et al., 1989; Halliwell, 1992). Because hydrogen peroxide is not exceptionally damaging to biological tissue, chelation of iron with desferrioxamine (DFO) would be expected to protect the tissue from any free radical effects. Iron, by itself, has been reported to elicit a free radical response from neural tissue (Willmore et al., 1983; Willmore and Rubin, 1984; Anderson and Means, 1985; Sadrzadeh et al., 1987; Subbarao and Richardson, 1990; Willmore and Triggs, 1991). The present study explores the free radical and iron sensitivity of the cytoplasmic pool of excitatory neurotransmitter. For this purpose we used the nonmetabolized excitatory amino acid, D-[^{3}H]aspartate. Release of D-[^{3}H]aspartate from synaptosomes has been found to be calcium independent (Wheeler, 1984; Levi and Gallo, 1986; Dunlop et al.,

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Abbreviation used: DFO, desferrioxamine.

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1989; Nicholls, 1989) in some systems. It has been shown to cause the displacement of endogenous glutamate from the cytoplasmic pool with minimal change in calcium-dependent release of glutamate (Nicholls and Sihra, 1986; Terrian et al., 1991). In addition, D-aspartate is not transported into synaptic vesicles in vitro (Naito and Ueda, 1985). This excitatory amino acid, therefore, allows us to target our evaluation to nonvesicular release mechanisms.

MATERIALS AND METHODS

Animals

Adult male Hartley guinea pigs (200–400 g) were housed under a 12-h light/dark cycle and provided with commercial guinea pig chow and water ad libitum. Research was conducted according to the principles enunciated in the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources, National Research Council.

Materials

Radioactive D-[2,3-^{3}H]aspartate (specific activity 15–25 Ci/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.). All other chemicals were obtained from Sigma Chemical (St. Louis, MO, U.S.A.). All solutions were prepared with double-distilled water.

Synaptosome isolation procedure

Guinea pigs were killed by cervical dislocation under isoflurane anesthesia. The brains were removed and cerebral cortices dissected out. The isolated cerebral cortex from each brain was placed in 10 volumes of ice-cold 0.32 *M* sucrose buffered with 5 m*M* Tris, pH 7.4. Synaptosomes were prepared using a modification of the discontinuous density gradient centrifugation method of Dodd et al. (1981) as described previously (Gilman et al., 1986*a*). Centrifugations were performed at 3°C in an ultracentrifuge (model OTD75B, Sorvall, Wilmington, DE, U.S.A.) using a w2/dt digital integrator to obtain consistent centrifugation. The tissue was dispersed with a motor-driven glass homogenizer and the initial homogenate centrifuged at 1,100 *g* for 5 min to yield a crude nuclear pellet and a low-speed supernatant. The supernatant was recovered and centrifuged at 17,000 *g* for 10 min to yield a mitochondrial pellet containing synaptosomes. The pellet was resuspended in 0.32 *M* sucrose and layered over a precooled discontinuous Ficoll gradient (7.5–13%) and centrifuged at 26,000 *g* for 30 min. After centrifugation, the middle band containing the purified synaptosomes was removed, diluted with sucrose, and pelleted at 11,500 *g* for 10 min.

Radioisotope uptake (loading)

The method used for radioactive amino acid loading of the synaptosomes was a modification of the superfusion method of Raiteri et al. (1974) as described previously (Gilman et al., 1986*b*, 1987). The final synaptosome pellet was suspended in 10 volumes of a buffer medium consisting of a high-sodium, calcium-free solution. The composition was as follows (in m*M*): NaCl 145, KCl 5, $MgCl_2$ 3.7, KH_2PO_4 1.2, Tris-HCl 20, glucose 10, pH 7.4. One-milliliter aliquots (containing 2–4 mg of protein/ml) of the synaptosome suspension were incubated at 37°C for 15 min to allow functional and metabolic equilibration. The synaptosome suspension was then loaded for 5 min with D-[2,3-^{3}H]aspartate (0.6 μCi/ml).

Amino acid neurotransmitter release

To evaluate the effect of free radicals on excitatory amino acid transmission in the cerebral cortex, a 1.0-ml aliquot of the radioisotope-loaded synaptosome suspension was placed on a filter unit consisting of a 0.45-μm nylon membrane filter positioned on a multiperforated support of a 10-ml perfusion chamber. The outflow of the perfusion chamber was then connected to a peristaltic pump. Using the highest pump speed (3 ml/min), a filter was washed for 15 min to remove unbound radioactivity. Wash medium consisted of buffer medium with or without (in calcium-free experiments) 1.2 m*M* $CaCl_2$. $MgCl_2$ was always adjusted to maintain osmolarity. During the washout period, the perfusate was either wash medium alone or wash medium containing 0.01% hydrogen peroxide with 0.1 m*M* ferrous sulfate. Solution in the chamber was replenished every 3 min. At the end of the 15-min wash period, a line from the peristaltic pump was aligned over a scintillation vial. Two wash perfusates containing 0.5 ml each of perfusate were collected directly into scintillation vials. Ten milliliters of a 6.2 m*M* K^+–containing, nondepolarizing, or a depolarizing, 56.2 m*M* K^+–containing efflux medium was then rapidly poured over the filter unit. The composition of the efflux medium was as follows (in m*M*): NaCl 145 or 95, KCl 5 or 55, $CaCl_2$ 1.2 (or 0), $MgCl_2$ 2.5 (or 3.7), KH_2PO_4 1.2, Tris-HCl 20, glucose 10, pH 7.4. The efflux medium was free of peroxide and of iron. Eight fractions containing 0.5 ml each of perfusate were collected every 10 s directly into scintillation vials.

To evaluate the effects of DFO, all solutions during preparation and wash of the synaptosomes contained 1.0 m*M* of the chelator to ensure adequate intracellular distribution. DFO was not present in the efflux medium. Handling of the synaptosomes and collection of fractions were otherwise identical to that described above.

Assay of samples

Ten milliliters of Biofluor was added to each scintillation vial and radioactivity determined for each perfusate fraction in a Beckman LS5801 liquid scintillation spectrometer with internal standardization, background correction, and automatically computed dpm relative to a external quench curve. The radioactivity remaining on the filters at the end of the superfusion was also calculated. Each filter was removed to a scintillation vial and the tissue solubilized with 1% sodium dodecyl sulfate before counting.

Expression of results

Fractional efflux of neurotransmitter was expressed as a percentage of total radioactivity, i.e., efflux = (dpm in filtrate × 100)/total radioactivity, where total radioactivity was the sum of all fractional filtrate dpm values and dpm remaining on the filter.

The tissue from only one guinea pig was used in synaptosome preparation on any given day. At least four preparations were used for each experiment. To remove the influence of basal release from high-potassium–evoked release, the average values obtained in normal potassium were subtracted from the average values obtained in high potassium and the standard error of the difference was calculated. The resulting difference curve was then offset to a final wash value of zero by subtracting a constant from every data

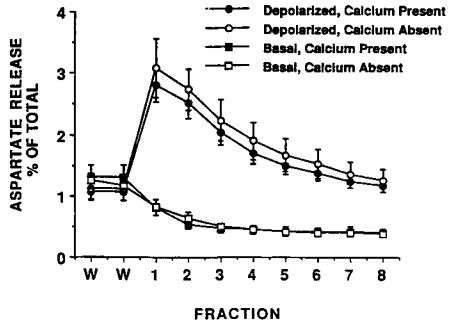

FIG. 1. Release of D-[^{3}H]aspartate from guinea pig cerebral cortical synaptosome fractions. Closed circles, depolarized, calcium present (n = 9); open circles, depolarized, calcium absent (n = 9); closed squares, nondepolarized, calcium present (n = 10); open squares, nondepolarized, calcium absent (n = 8). After preloading with D-[^{3}H]aspartate and washing, synaptosome aliquots were perfused with a low K^+ medium for 20 s (W, W), then either 6.2 m*M* or 56.2 m*M* K^+-containing medium with or without calcium was similarly perfused for 80 s (fractions 1–8). Each point represents mean and SEM of all experiments.

point. Statistical differences were evaluated with Student's *t* test with the Bonferroni correction for multiple comparisons when appropriate. Significance was accepted at $p < 0.05$.

RESULTS

D-[^{3}H]Aspartate release by synaptosomes

Figure 1 shows the time course of D-[^{3}H]aspartate release under resting nondepolarized conditions and under depolarized (high K^+)-evoked release. As can be seen (Fig. 1, closed circles), application of a high K^+ medium containing 1.2 m*M* Ca^{2+} initially induced a two- to threefold increase in the release of D-[^{3}H]aspartate by the synaptosome preparation. The evoked release declined gradually and approached resting (nondepolarized) levels within 80 s. To determine the extent of calcium dependency of D-[^{3}H]-aspartate release, synaptosomes were perfused with a depolarizing, high K^+ calcium-free medium, with $MgCl_2$ replacing the omitted $CaCl_2$. The removal of calcium from the depolarizing medium (open circles) had no effect on D-[^{3}H]aspartate release, indicating that high K^+–induced release is not calcium dependent ($p > 0.05$). Basal release (squares) declined steadily throughout the experimental period. Removal of calcium from the nondepolarizing medium had no effect on basal D-[^{3}H]aspartate release ($p > 0.05$) (Fig. 1, closed squares vs. open squares).

Basal release of D-[^{3}H]aspartate

Pretreatment of synaptosomes with 0.01% hydrogen peroxide enhanced basal release of D-[^{3}H]-aspartate compared with untreated controls. Release was increased significantly throughout the experimental period ($p < 0.05$) (Fig. 2). To determine whether the actions of peroxide were through Fenton-generated hydroxyl radicals, we tested the iron chelator DFO. DFO pretreatment had little effect of its own but prevented the peroxide enhancement of basal release (Fig. 2). These actions were independent of the calcium in the external media. The same pattern was observed in calcium-free solution (Fig. 2B).

To evaluate the role of iron in the peroxide-induced enhancement of basal release, we evaluated the effects of 0.1 m*M* ferrous sulfate alone and in combination with peroxide. Treatment with iron caused a significant increase in release ($p < 0.05$) that was comparable with that of peroxide treatment (Fig. 3). The effects of iron and peroxide were not additive. Release after treatment with both iron and peroxide was not different from the release after exposure to either alone. Removal of calcium from the media (Fig. 3B) did not alter the enhancement of D-[^{3}H]aspartate release by iron or iron with peroxide.

Figure 4 summarizes the effects on basal release. The total release of D-[^{3}H]aspartate was calculated from the sum of the percentage released during the 10 fractions for each experiment. It can be seen that the effects were calcium independent. Total release did not differ with the presence (open bars) or absence (filled bars) of calcium. The aspartate release elicited after treatment with peroxide (16.8 ± 3.7% Ca^{2+} absent, 17.5 ± 1.9% Ca^{2+} present), iron (15.6 ± 2.0%,

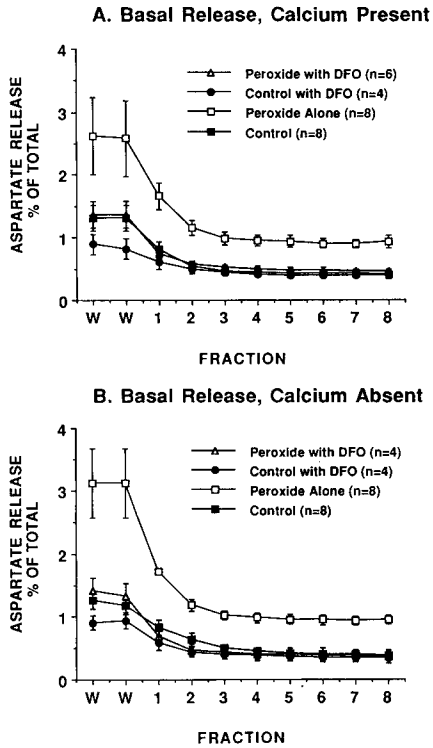

FIG. 2. Inhibition of peroxide effects on basal D-[^{3}H]aspartate release in the presence of DFO. **A**: In the presence of calcium. **B**: In the absence of calcium. Each point represents mean and SEM of all experiments. The number of experiments is indicated in the figure key.

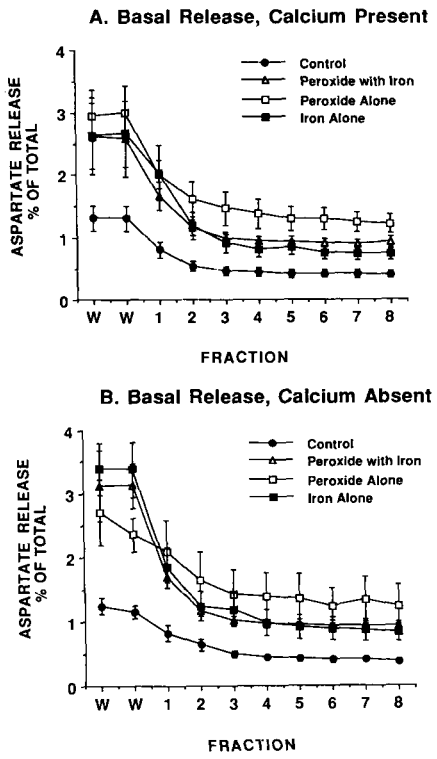

FIG. 3. Effect of peroxide with iron, peroxide alone, or iron alone on spontaneous, nondepolarized (5 m*M* K^+-evoked) release of D-[^{3}H]aspartate. **A**: In the presence of calcium; each point represents mean and SEM of all (n = 8) experiments. **B**: In the absence of calcium; each point represents mean and SEM of all (n = 8) experiments.

Ca^{2+} absent, 13.3 ± 2.0% Ca^{2+} present), and peroxide plus iron (14.9 ± 1.5% Ca^{2+} absent, 13.6 ± 1.6% Ca^{2+} present) were comparable and statistically significant compared with untreated controls ($p < 0.05$). DFO

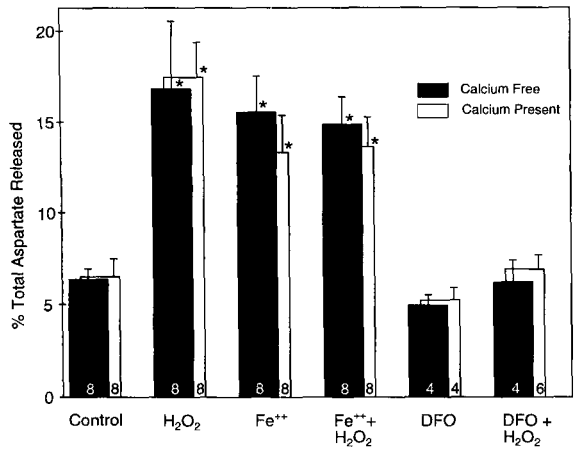

FIG. 4. Summary of effects of peroxide with iron, peroxide alone, or iron alone on basal D-[^{3}H]aspartate release. Each bar represents the sum of percent release for 10 fractions (fractions W–8). The number of experiments is indicated at the base of each bar. Open bars, in the presence of calcium; filled bars, in the absence of calcium. *$p < 0.05$, compared with control.

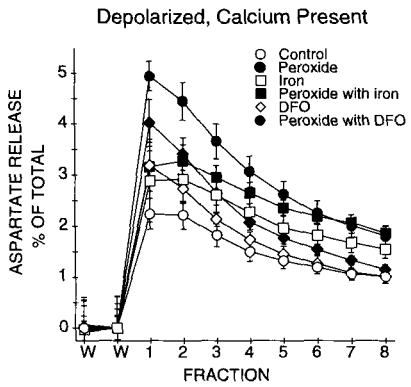

FIG. 5. Effect of peroxide, iron, and DFO on depolarized (high K^+-evoked) release of D-[^{3}H]aspartate from cerebral cortical synaptosomes in the presence of peroxide. Circles, control and peroxide alone (n = 8); squares, iron and peroxide with iron (n = 8); diamonds, DFO and DFO with peroxide (n = 5). Each point represents mean and SEM of all experiments.

pretreatment was capable of preventing the peroxide-induced increase in release (6.2 ± 1.2% Ca^{2+} absent, 6.9 ± 0.8% Ca^{2+} present).

Evoked release of D-[^{3}H]aspartate

Exposure of isolated presynaptic nerve terminals to 0.01% peroxide caused a significant increase in evoked D-[^{3}H]aspartate release in the presence of calcium ($p < 0.05$) (Fig. 5, circles). The enhancement by iron alone was not significant and peroxide was ineffective in the presence of the metal (Fig. 5, squares). Results with DFO were similar to those with iron. Release after DFO treatment was not statistically different from control. In the presence of DFO, peroxide no longer increased release (Fig. 5, diamonds). In the absence of calcium, high K^+-evoked release of D-[^{3}H]-aspartate was not significantly altered by any of the treatments (data not shown).

DISCUSSION

We have shown that free radicals increase the basal release of D-[^{3}H]aspartate from cortical synaptosomes. The D-isomer of aspartate is transported into the synaptosomes through the same high-affinity transporter as L-glutamate and L-aspartate (Balcar and Johnston, 1972; Drejer et al., 1983) and is metabolically less reactive. However, D-aspartate is not transported into synaptic vesicles (Naito and Ueda, 1985) and so only labels the cytoplasmic pool. As would be expected under this condition, release of D-aspartate from synaptosomes has been shown to be calcium independent (Wheeler, 1984; Levi and Gallo, 1986; Dunlop et al., 1989; Nicholls, 1989). A calcium-dependent component of D-aspartate release, however, has been observed in slice and neuronal preparations (Drejer et al., 1983; Levi and Gallo, 1986; Palaiologos et al., 1989; Pellegrini-Giampietro et al., 1990; Westergaard et al., 1991; Belhage et al.,

1992). In our preparation of cortical synaptosomes, both the basal and evoked release of D-aspartate was independent of calcium, suggesting a nonvesicular compartmentalization of the amino acid.

Increased basal release

The present study demonstrates that peroxide significantly increases basal, nondepolarized release of D-[^{3}H]aspartate from the cytoplasmic pool. Similarly, Pellegrini-Giampietro et al. (1990) reported an increase in basal release of excitatory amino acids from rat hippocampal slices with free radical exposure. In addition, an electrophysiological study (Colton and Gilbert, 1986) showed an increase in spontaneous transmitter release at the lobster neuromuscular junction after treatment with hydrogen peroxide. In the present experiments, DFO, an iron chelator, prevented the stimulatory effect of peroxide, maintaining basal release at control levels. In contrast, iron enhanced basal release to an extent comparable with that of peroxide. The actions of peroxide and iron on basal release were not additive.

Peroxide generates highly reactive, tissue-damaging hydroxyl free radicals through an interaction with intrinsic tissue iron as well as with extrinsically added iron (i.e., the Fenton reaction; Halliwell, 1992). Chelation of iron with DFO would be expected to prevent free radical formation. Our observation that DFO prevents the peroxide-induced increase in release of D-[^{3}H]aspartate demonstrates that the actions of peroxide are mediated via the Fenton reaction and that peroxide, by itself, is not effective. Because providing additional iron does not potentiate the actions of peroxide alone, the Fenton reaction may be already proceeding at a maximal rate. Alternatively, because hydroxyl radicals do not travel far from their site of formation, the added iron might not gain access to the appropriate location to promote further damage.

Iron is toxic to neural tissue. Injection of iron salts into cortex induces epileptiform discharges and recurrent seizures (Willmore et al., 1978, 1986; Reid and Sypert, 1980). Iron can also elicit parkinsonian-like symptoms when injected into the substantia nigra (Ben-Shachar and Youdim, 1990). It has been suggested that iron exerts these actions through generation of free radicals. Willmore et al. (1983) reported the production of superoxide with injections of iron into rat cortex. In addition, in brain tissue, iron initiates lipid peroxidation, a consequence of free radical exposure (Willmore et al., 1983; Willmore and Rubin, 1984; Anderson and Means, 1985; Sadrzahdeh et al., 1987; Subbarao and Richardson, 1990; Willmore and Triggs, 1991). Antioxidants such as dimethyl sulfoxide (Willmore and Rubin, 1984), mannitol (Anderson and Means, 1985), methylprednisolone (Anderson and Means, 1985), α-tocopherol (Willmore and Rubin, 1984; Hall et al., 1991), the new 21-amino steroids (Hall et al., 1991), as well as DFO (Sadrzadeh et al., 1987; Subbarao and Richardson, 1990) are effective in preventing iron-induced lipid peroxidation. In this light, our observation that iron by itself increased the basal release of D-[^{3}H]aspartate was of particular interest. The actions of iron were indistinguishable from the actions of peroxide and the effects of the two agents were not additive at the concentrations used, suggesting a common path of action.

Possible mechanisms for increasing basal release

Although the mechanism of basal release of amino acid neurotransmitters has not yet been resolved, many of the processes that influence release from this cytoplasmic pool (Bernath, 1991) could be sites for modulation by free radicals. A nonspecific change in membrane permeability is unlikely because no change in membrane resistance measured electrophysiologically results from exposure to peroxide, iron, or their combination (Pellmar, 1987). Basal release would be expected to increase with membrane depolarization. Yet because peroxide does not affect membrane potential of hippocampal neurons (Pellmar, 1987), this is also an unlikely mechanism. Peroxide-induced free radicals have been shown to disrupt sodium–calcium exchange (Hayashi et al., 1989), which would increase intracellular calcium concentration and consequently increase neurotransmitter release. Alternatively, the radicals could target amino acid transport mechanisms preventing neuronal reuptake of the amino acid. This possibility is supported by a recent finding that free radicals block glutamate uptake in cultured glial cells (Volterra et al., 1992). Basal release is also affected by changes in intracellular cyclic AMP levels and activation of cyclic cAMP-dependent protein kinase A (Browning et al., 1985; Schoffelmeer et al., 1985; Ghirardi et al., 1992). Free radicals might influence release through these second messenger systems.

Evoked release

Treatment with peroxide significantly increased evoked release of D-[^{3}H]aspartate in the presence of calcium but not in its absence. Yet we have shown that all of the evoked release of D-[^{3}H]aspartate can be explained by a calcium-independent process. It appears, therefore, that calcium can influence the mechanism through which peroxide alters this process. DFO and iron did not have the predicted actions on the evoked release, suggesting that the process is not mediated through a free radical mechanism.

Calcium-independent evoked and basal release are likely to be two distinct mechanisms. Not only are they differentially affected by free radicals as we observed in this study, but also by the oxidizing agent, chloramine-T (Gilman et al., 1993). Basal release is clearly sensitive to an oxidation reaction, whereas calcium-independent evoked release does not share this sensitivity. The underlying mechanism of the calcium-independent evoked release is, as yet, unknown (Bernath, 1991).

Calcium-dependent and independent evoked release respond differently to free radical exposure. The calcium-dependent release of L-[^{3}H]glutamate is significantly reduced by hydrogen peroxide, whereas the calcium-independent evoked release is minimally affected (Gilman et al., 1992). Because the calcium-independent release of L-glutamate was evaluated in calcium-free solution, these results are directly comparable with the absence of effect of peroxide on the evoked release of D-aspartate in the absence of calcium. Because L-glutamate distributes to both the cytoplasmic and vesicular pools whereas D-aspartate only enters the cytoplasmic pool of excitatory amino acids, we can also evaluate the calcium-independent evoked release of D-aspartate in the presence of calcium. Under these conditions, we observed an increase in release. Although this was unexpected, it is not inconsistent with the decrease in L-glutamate release in the presence of calcium, because different pools of transmitter are being evaluated. These two pools of neurotransmitter are differentially affected by exposure to free radicals probably through effects on distinct cellular targets.

Relevance to ischemic injury

Oxygen free radicals are thought to be involved with the neuronal damage resulting from ischemia/reperfusion (Demopoulos et al., 1982; Hall and Braughler, 1989). The effects of ischemia and free radical exposure share many common features. As we report here for free radical exposure, hypoxic injury causes the extracellular accumulation of excitatory amino acids in vivo (Benveniste et al., 1984; Drejer et al., 1985; Hagberg et al., 1985; Ikeda et al., 1989; Shimada et al., 1993) as well as in vitro in the brain slice (Bosley et al., 1983; Ikeda et al., 1989; Pellegrini-Giampietro et al., 1990) and synaptosomal (Sanchez-Prieto and Gonzalez, 1988; Rubio et al., 1991) preparations. In most (but not all; see Drejer et al., 1985) of these studies the ischemia-induced increase in extracellular transmitter was calcium independent (Sanchez-Prieto and Gonzalez, 1988; Ikeda et al., 1989; Pellegrini-Giampietro et al., 1990; Rubio et al., 1991). Similarly, our results demonstrate that the enhanced basal release after free radical exposure is independent of extracellular calcium. In the synaptosomal preparation, calcium-dependent evoked release of neurotransmitter was reduced with a model of ischemia (Sanchez-Prieto and Gonzalez, 1988) as we observed in an earlier study with peroxide treatment (Gilman et al., 1992). It is likely that free radicals and ischemic injury produce these similar actions through a common pathway (see also Pellegrini-Giampietro et al., 1990).

Conclusion

In conclusion, our results suggest a complex action of hydroxyl free radicals at the presynaptic nerve terminal. Hydroxyl free radicals generated by hydrogen peroxide may be exerting multiple effects on several neuronal components involved in excitatory amino acid neurotransmitter release. These effects manifest as an apparent increase in nonvesicular basal release and a decrease in depolarization-induced vesicular release. This series of events would be expected to significantly alter neuronal activity and to result in the dysfunction of integrated neural networks.

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